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Long term diazotrophic cultivation induces phycobiliprotein production in *Anabaena variabilis* IMU8

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Abstract: Cyanobacteria are considered as a sustainable feedstock for the production of biochemically active compounds such as phycobiliproteins (PBPs). In this study, the impact of nitrogen (N) and phosphorus (P) availability on PBP production of "N-free acclimated" *Anabaena variabilis* IMU8 was analyzed. Upon isolation and identification, the cyanobacterium has been maintained in N-free BG-11 medium for more than 20 months. For experimentation, the strain was incubated in N-replete, N-depleted, N-P-depleted BG-11 medium. Long-term diazotrophic cultivation of *A. variabilis* IMU8 resulted in elevated PBP productivity with a limited impact on growth. When compared to N-depleted ones, N supply stimulated a slight induction of growth and total saccharide production, but total protein content did not change while PBP production decreased. On the other hand, N-P-depletion resulted in decreased growth rate along with reduced total protein and PBP production while rapid induction of total saccharide production was recorded. Fourier transform infrared spectroscopy results refer that membrane-bound oligosaccharides may have regulatory roles for PBP production in *A. variabilis* IMU8 during long term diazotrophic cultivation.

Introduction

Cyanobacteria are ancient group of oxygenic photosynthetic prokaryotes which are well adapted to survive in different habitats of oceans, seas, rivers, fresh and alkaline waters, humid rocks, caves, sands, deserts, brackish waters, snows, glaciers as well as hot spring water resources (Pandey *et al.*, 2013; Bolay *et al.*, 2018). Unlike most other photosynthetic organisms, cyanobacteria are able to make photosynthesis efficiently in the low chlorophyll absorption spectrum by means of phycobiliproteins (Glazer *et al.*, 1986). Phycobiliproteins (PBPs) are water-soluble fluorescent pigment-protein complexes of photosynthesis machinery in cyanobacteria, some red algae, and cryptomonads which facilitate light absorption over the wavelength range 450 to 655 nm (Manirafasha *et al.*, 2016). The major classes of PBPs are phycoerythrin (PE, λA max = 540-570 nm; λF max = 575-590 nm), phycocyanin (PC, λA max = 610-620 nm; λF max: 645-653 nm) and allophycocyanin (APC, λA max = 650-655 nm; λF max = 657-660 nm) (Khazi *et al.*, 2018). The cyanobacterial PBPs find a wide range of use in biomedical research, food, cosmetics, and pharmaceutical industries as fluorescent labels, coloring reagent, and drug additive owing to their highly antioxidant nature (Lau *et al.*, 2015; Sonani *et al.*, 2016; Khan *et al.* 2019).

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Phycobiliprotein content of cyanobacteria shows strain-specific differentiation (Singh *et al.*, 2005). Moreover, PBP production is dynamically regulated in a cyanobacterium as a response to changes in environmental factors such as pH, temperature, salinity, irradiance and nutrient availability (Fatma *et al.*, 2009; Li *et al.*, 2019). In aquatic ecosystems, N and P availability are the main factors that limit cyanobacterial growth (Peng *et al.*, 2016). In this study, three different cyanobacteria isolated from Hıdırlar thermal spring located in Çanakkale province in Turkey were evaluated for their PBP production, and some physiological changes in best PBP producing strain were analyzed in relation to nitrogen and phosphorus availability.

Material and Methods

Isolation and identification of the strains

The strains *Anabaena variabilis* IMU8 and *Nodularia sp.* IMU17 were isolated from Hıdırlar thermal spring (39°89'N 27°17'E) located in Çanakkale, and *Nostoc carneum* IMU11 was isolated from a paddy field (40°85'N 26°32'E) located in Edirne province of Turkey in March 2017. The strains were identified based on morphological characteristics (Waterbury *et al.*, 2006; Rajaniemi *et al.*, 2005) and genomic information. The strains have been cultured and maintained in Istanbul Medeniyet University Microalgae Culture Collection, Istanbul Medeniyet University, Turkey.

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In order to obtain genomic information for identification of the strains, the genomic DNA fragment was amplified by PCR, sequenced, and analyzed according to (Khazi *et al*., 2018; Lu *et al*., 1997). DNA amplification from genomic DNA containing a partial 16S ribosomal RNA region was performed with PCR by using the following primers: Forward (27F): 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse (809R): 5'-GCTTCGGCACGGCTCGGGTCGATA-3'. The same primers were used for Sanger sequencing. Sequence comparison of the 16S rRNA genes was performed using the NCBI databases with BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit-graphical biological sequence editor v7.0.9. Based on Sanger sequencing information, the strains were registered to NCBI with accession numbers; MK928972.1 (*A. variabilis* IMU8), MK929013 (*N. carneum* IMU11), and SUB6443578 (*Nodularia* sp. IMU17).

Inoculum preparation

The cyanobacteria used in this study are all diazotrophic cyanobacteria showing good growth ability in N-lacking BG-11 growth medium. After isolation in March 2017, strains have been maintained in N-lacking BG-11 growth medium. The cultures were refreshed every 15-20 days for storage. Thus, the strains used in this study can be considered as acclimated to N-deprivation for 20 months until the experimentation. Thereby, cells grown in N-lacking BG11 medium were used as the experimental reference for PBPs production. For experimentation, exponentially growing cells were harvested by means of centrifugation at 1000 rpm for 2 min, the cell pellet was washed two times with distilled water and used as inoculum (5% v/v). Cyanobacteria were cultivated in 250 mL flasks containing 100 mL of N-lacking BG-11 growth medium on a temperature-controlled (27 ± 1°C) orbital shaker (Sartorius, Certomat BS-T, USA) at 120 rpm under the continuous illumination of 100 μE/(m^2/s). For evaluation of P and N availability on PBPs production, cultures were incubated in N-replete, N-deficient, or N-P-deficient BG11 growth medium.

Biomass production

The biomass production rate of cyanobacteria was determined based on changes in Chlorophyll-*a* concentration. The concentration of Chl-*a* was calculated according to (Zavřel *et al*., 2015) with slight modifications. Each sample (2 mL) was centrifuged for 5 min at 10000 *g*, and the pellet was resuspended in 1.5 mL of methanol (100%, precooled to 4°C), vortexed for 1 min and placed on a rotator for mixing for 20 min under room temperature. Then samples were centrifuged for 5 min at 10000 g, and the absorbance of the supernatant was measured at OD_{665} and OD_{720} by using methanol as a blank. The concentration of Chl-*a* was calculated according to the following equation (Ritchie *et al*., 2007).

Extraction and estimation of phycobiliproteins

Five mL of culture was centrifuged at 4000 g for 5 min. The pellet was resuspended in 5 mL of sodium phosphate buffer (0.1 M, pH 7.0, containing 1 mM sodium azide) and sonicated (200 W, 30 kHz) for 2 min followed by a freeze-thaw process. The suspension was centrifuged at 4000 *g* for 5 min, and the clear supernatant was collected for absorbance measurement. The absorbance of the supernatant was measured at OD_{562}, OD_{620}, and OD_{652} for calculation of the Phycobiliproteins-PBPs (Phycocyanin-PC, Allophycocyanin-APC, Phycoerythrin-PE) according to the following equations (Bennett *et al*., 1973).

Quantification of total saccharide and total protein content

Each sample (5 mL) was pelleted by centrifugation (2000 g, 5 min), washed with sterile water, and centrifuged again (4000 g, 5 min). The pellet was transferred into a 1.5 mL preweighed centrifuge tube. After another centrifugation step, the supernatant was completely removed, and the remaining cell pellet was first incubated (open cap) at 40°C in a thermomixer for 30 min and weighed for fresh weight determination. Then the cell pellet was completely dried at 80°C in a thermomixer overnight. The dry weight of the cells was determined by the weight of the cell tubes subtracted from the dead weight of the tube. Total saccharide and total protein content of the cells were calculated based on the dry weight.

The cell pellet obtained after Chl-*a* extraction was used for quantification of total carbohydrates by means of the modified phenol-sulfuric acid method as described by (Zavřel *et al*., 2018).

For protein quantification, lyophilized cell pellets were re-suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 2% SDS, 10 mM EDTA, and protease inhibitor mix), subjugated to sonication (3510E-DTH, Branson) for 1 min at 60% power (7 W/pin) and centrifuged at 13000 *g* at 4°C. The supernatant was then used for protein determination with the Bicinchoninic Protein assay (He *et al*., 2011).

Fourier Transform Infrared Spectroscopy (FTIR)

Approximately 50 mg of lyophilized cell biomass was vacuum-dried at 40°C for 1 h, and the dried sample was placed on the sampler module. Infrared spectra were recorded over a wave number range of 4000 to 400 cm^{-1} with 128 scans on a Fourier transform infrared spectroscopy (Perkin Elmer-L160000A, USA) equipped with an ATR module.

Each experiment was repeated twice with three biological replicates. Thus, the final data in this article are the mean values of at least three separate samples collected at two different times (n = 6). Means of averages with standard errors are presented throughout the manuscript, and data evaluation was done by using *t*-tests (two tails, pair type) with the significance criteria of 0.05 to assess the significance between different groups evaluated for the same time point.

Results and Discussion

Phycobiliprotein (PBP) production potential of long-therm N-deprived strains

Cyanobacteria and photosynthetic eukaryotes possess similar composition of photosystem (PS) I and II; however, phycobilisomes containing major light-harvesting proteins in PSI of cyanobacteria facilitate efficient photosynthesis in the low chlorophyll absorption spectrum (Glazer *et al*., 1986). The phycobilisomes are made up of phycobiliproteins (PBPs), which can be also utilized as nitrogen reservoirs under unfavorable environmental conditions (Schwarz *et al*., 2005). Thereby, the content and composition of PBPs

differentiate depending on the evolutionary characteristics of the strain and fluctuation in environmental conditions (Li *et al*., 2019). In this study, the strains have been maintained in N-lacking BG-11 medium for approximately 20 months. Thus, the strains can be considered as "N-free acclimated" diazotrophic strains. In order to check their PBP content, the strains were incubated in liquid N-lacking BG-11 growth medium, and the PBP contents were measured 6 days after inoculation when they all were in the exponential growth phase. The strain *Anabaena variabilis* IMU8 produced the maximum amount of PBP equivalent to 17.3% of the dry weight while it was 13.1% and 4.9% in *Nostoc carneum* IMU11 and *Nodularia sp* IMU17 respectively (Fig. 1). The PC and APC were dominant phycobiliproteins in *A. variabilis* IMU8. The PC, APC, and PE contents were measured as 7.7%, 6.3%, and 3.3% in *A. variabilis* IMU8. On the other hand, the PC, APC, and PE contents of *A. variabilis* IMU8 was approximately 20.3%, 5%, and 68.7% higher than those of *N. carneum* IMU11, and 70.7%, 64.7%, and 89.5% higher than those of *Nodularia sp.* IMU17. Therefore, *A. variabilis* IMU8 was selected for further analysis. Earlier research showed that freeze-dried samples of *Spirulina* sp. contain maximum phycobiliprotein content of 22.5% (w/w), *Phormidium* sp. and *Lyn-gbya* sp. contain 5.4% (w/w) and 5.8% (w/w), respectively (Patel *et al*., 2005). Thereby 17.3% (w/w) PBP content of *A. variabilis* IMU18 is compatible with those of *Spirulina* sp.

Anabaena variabilis IMU8 is heterocyst-forming diazotrophic filamentous cyanobacterium. Vegetative cells are cylindrical, heterocysts form in a regular pattern in the filament, and akinetes are oval or elliptical without any connection with heterocysts. The filaments are mostly curved or entangled when cultivated in N-replete medium

Growth in response to N and P availability

Nitrogen and P are the main nutrients that affect microalgal and cyanobacterial growth in water ecosystems (Paerl *et al*., 2016). Decreased level of N and P was reported to stimulate diazotrophic cyanobacterial growth while causing decreased growth of eukaryotic microalgae in stream water (Mulholland *et al*., 1995). In order to see time-dependent changes in growth, PBP production, and related parameters in *A. variabilis* IMU8 in response to N- and P availability, the strain was incubated in N-replete, N-deficient, and N-P-deficient BG11 growth medium for 16 days of the incubation period (Fig. 3). There was no significant change in first 10 days of incubation in N-replete or N-deficient conditions; however, when compared to N-depleted cells, approximately 26% increase in growth was observed on the 12th day in N-replete cells and ended up with 20% increased growth at the end of 16 days of incubation. On the other hand, comparing to N-deprived ones, there was a gradual decrease in growth when *A. variabilis* IMU8 was incubated in the N-P-deficient growth medium. The decrease in growth was calculated as approximately 21.8% and ended up with a maximum of 60% decreased growth by the end of 16 days of the incubation period. Amongst nutrients, N and P are of special importance as mainly N availability affects C and N allocation to PBPs, and P availability affects akinete formation in filamentous cyanobacteria (Sarma and Khattar, 1992).

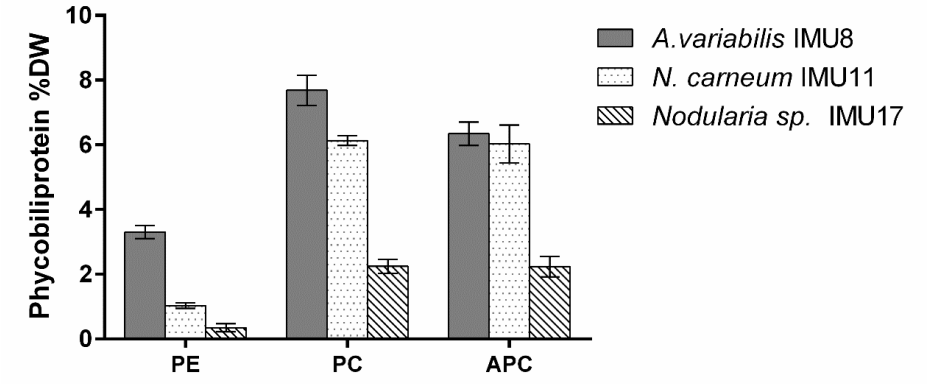

FIGURE 1. Phycobiliprotein content of cyanobacteria maintained in N-free BG-11 medium for 20 months. PBP, Phycobiliprotein; PE, Phycoerythrin; PC, Phycocyanin; APC, Allophycocyanin. For all data sets, each point represents the mean ± SD of at least three replicate culture flasks.

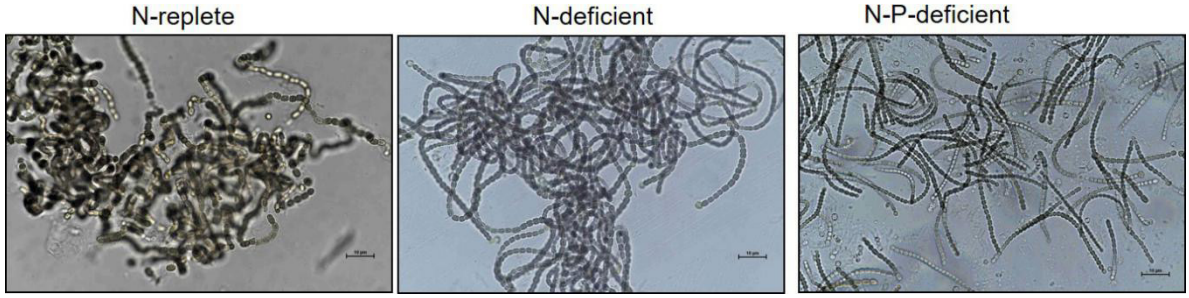

FIGURE 2. Microphotograph of *A. variabilis* IMU8 incubated in N-replete, N-deficient, and N-P-deficient growth conditions for 12 days.

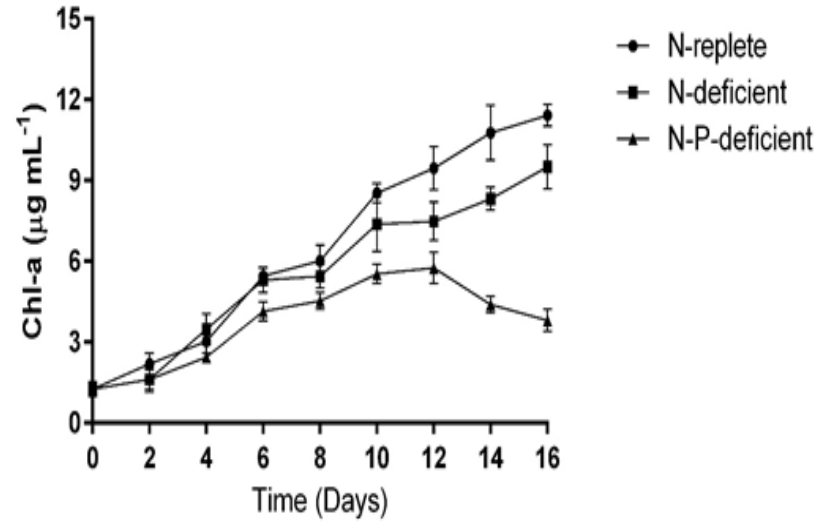

FIGURE 3. Change in growth of *A. variabilis* IMU8 incubated in N-replete, N-deficient, and N-P-deficient growth conditions.

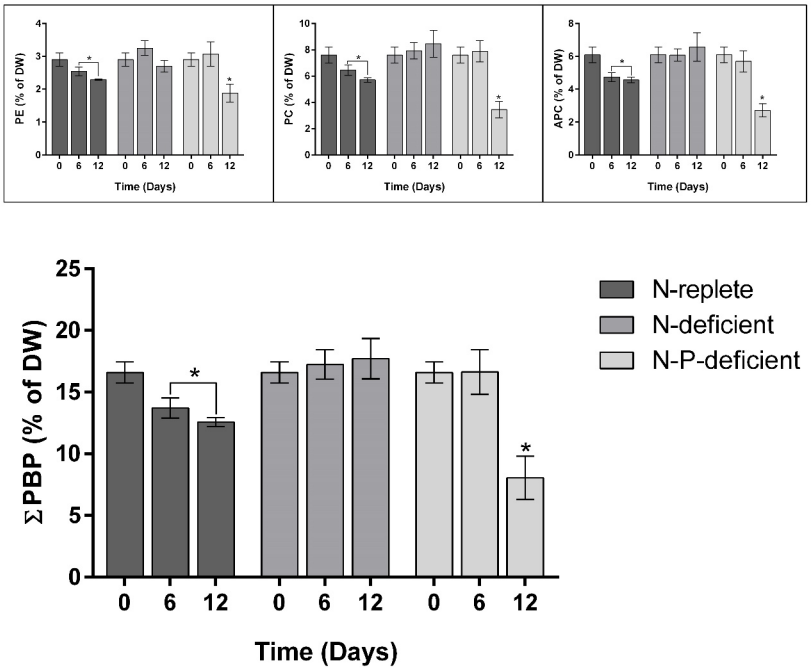

FIGURE 4. Phycoerythrin (PE), Phycocyanin (PC), Allophycocyanin (APC), and total Phycobiliprotein (PBP) content of *A. variabilis* IMU8 in response N and/or P availability**.** Asterisk (*) indicate statistical significance criteria of 0.05.

Phycobiliprotein production of A. variabilis IMU8 in response to N and P availability

The fluctuations in the ratios and amount of PBPs production in *A. variabilis* IMU8 were analyzed by harvesting cells on the 6th and 12th days of N-replete, N-deficient, and N-P-deficient cells (Fig. 4). Results showed that N-availability limits PBPs production. When compared to N-deprived ones, overall PBP equivalents production of N-replete cells decreased by approximately 20.4% and 29% on the 6th and 12th days of incubation. There was approximately 32.4%, 30.5%, and 14.7% decrease in PC, APC, and PE levels by the end of 12 days of incubation when *A. variabilis* IMU8 was incubated in N-replete medium. On the other hand, The PBP levels did not change in the first 6 days; however, 54.5% decrease was recorded on the 12th day of incubation. The decrease in PC, APC, and PE levels were recorded as approximately 59.1%, 58.6%, and 30.2% when *A. variabilis* IMU8 was incubated in N-P-deficient medium for 12 days.

It seems that P-deprivation results in decreased growth and PBP production. Thereby, P-deprivation might cause penalties in N-fixation processes that need intensive energy consumption. Nitrogenase activity of diazotrophic cyanobacteria was found to be closely related to P availability (Sarma and Khattar, 1992). When *A. variabilis* IMU8 was incubated in the N-P-deficient medium, total protein production decreased, and total saccharide production rapidly increased as compared to N-deprived ones (Fig. 5). The decrease in total protein content was calculated as 14.2 and 31.8% on the 6th and 12th days of incubation. On the contrary, when compared to N-deprived *A. variabilis* IMU8, the total saccharide content of N-P-deprived ones increased up to 42.7% and 109% on the 6th and 12 days of incubation. In cyanobacteria, P has been reported to be essential for cell growth, as it is a vital macromolecular constituent of phospholipids, proteins, polysaccharides, and cofactors (Peng *et al*., 2016). Rapid induction of saccharide production and degradation of cellular proteins and PBPs may refer that *A. variabilis* IMU8 undergo hibernation state by limiting growth

and producing storage carbon compounds in response to P-deprivation under diazotrophic cultivation.

Its ability to grow efficiently in the N-lacking growth medium and increased production of PBP may favor *A. variabilis* IMU8 as a potential candidate for large scale production of cyanobacterial PBP. Supportively, an *Anabaena* strain (*Anabaena* sp. NCCU-9) producing the highest PBPs amongst 18 different cyanobacterial isolates was reported, and it was highlighted that N-deprivation was most favorable for increased PBP production (Fatma *et al.*, 2009). Likewise, the N-free growth of diazotrophic *Nostoc* sp. was reported to increase biomass as well as cyanobacterial PC productivity (Lee *et al.*, 2017). The strain *A. variabilis* IMU8 employed in the current study can be considered as "N-free" acclimated diazotrophic filamentous cyanobacterium as it has been maintained under N-free condition over 20 months until the experimentation. When compared to N-deprived cells, N-supply did not cause a significant change in total protein production while there were slight increases in total saccharide production during 12 days of the incubation period (Fig. 5). There was approximately 19.7% and 25% increase in total saccharide production when *A. variabilis* IMU8 was incubated in the N-replete medium. Considering limited suppression of growth and total saccharide production, no visible effect on total protein production, and considerably higher PBP production under N-deprivation, results refer that long-term diazotrophic cultivation of N-fixing cyanobacterium may facilitate increased PPB productivity.

FTIR analysis

FTIR analysis was performed to see overall changes in biomass characteristics in response to N- and P- availability (Fig. 6). Infrared spectra were recorded in transmission mode with 128 scans in the range 4000-400 cm^{-1}. The bands were assigned to specific molecular groups on the basis of biochemical standards and published studies as previously described (Movasaghi *et al.*, 2008). The major bands observed in all groups were attributed to C-O stretching frequencies of the C-OH groups of polysaccharides (1045 cm^{-1}), Amide I absorption (1652 cm^{-1}), Amide II absorption (1544 cm^{-1}), asymmetric stretching vibration of acyl chains (2925 cm^{-1}), O-H stretching of carbohydrates and N-H stretching of proteins (3380 cm^{-1}).

On the other hand, some bands were clearly visible in N-deprived and N-P-deprived cells while they were lost in N-replete cells. The bands attributed to PO_2^- asymmetric stretching of phosphodiesters (1076 cm^{-1}), membrane-bound oligosaccharide C-OH bond (1145 cm^{-1}), and symmetric CH3 bending modes of the methyl groups of proteins (1401 cm^{-1}) were visible in N-deprived and N-P-deprived *A. variabilis* IMU8 while they were lost in N-replete cells. These bands might be related to systemic resistance. Membrane-bound oligosaccharides were described as biologically active elicitors at low concentrations (Albersheim *et al.*, 1992). In plants and algae, oligosaccharides were reported to undertake regulatory roles on growth, development, and defense against stressors including nutrient limitation (Courtois *et al.*, 2009). Our results refer that N-availability itself might be responsible for the induction of membrane-bound oligosaccharide production in the diazotrophic

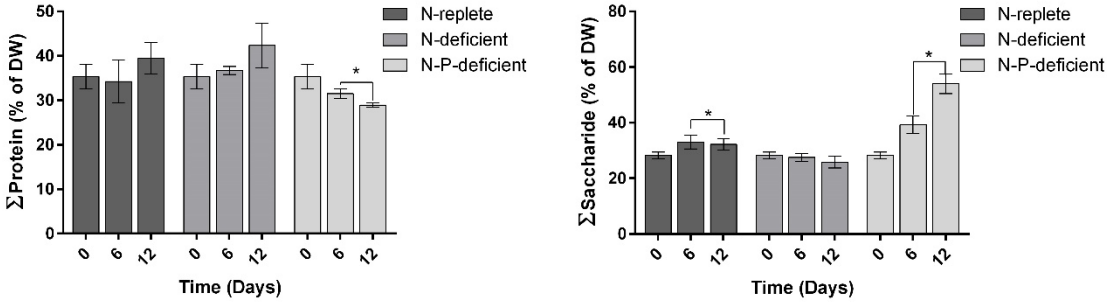

FIGURE 5. Change in total soluble protein and saccharide levels in *A. variabilis* IMU8. Asterisk (*) indicate statistical significance criteria of 0.05.

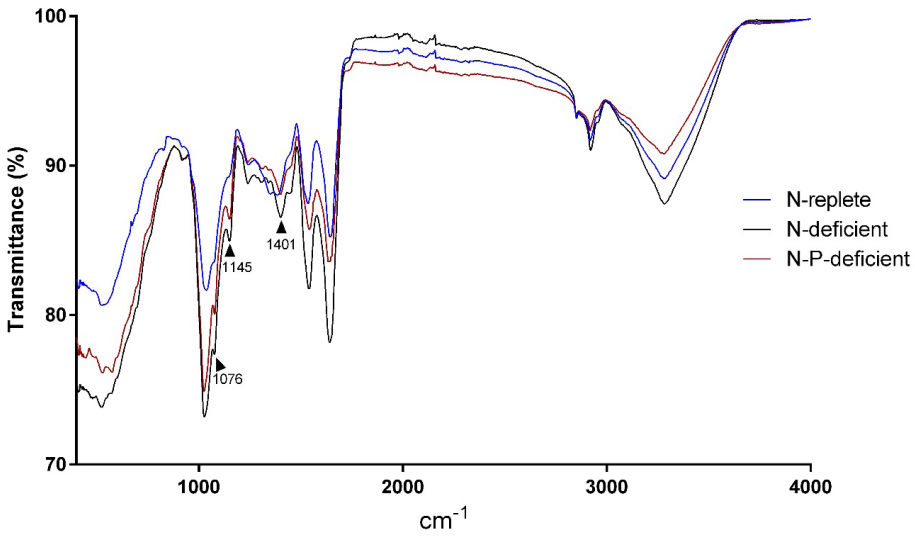

FIGURE 6. FTIR spectrum of 6-days old N-replete, N-deficient, and N-P-deficient *A.variabilis* IMU8.

Conclusion

As a conclusion, P deprivation of "N-free acclimated" *A. variabilis* IMU8 do not favor PBPs production. However, long-term diazotrophic cultivation of *A. variabilis* IMU8 resulted in elevated PBP productivity with a limited impact on growth. This approach might be employed for other PBP producing diazotrophic cyanobacteria. Moreover, membrane-bound oligosaccharides may have a regulatory role for PBP production in *A. variabilis* IMU8 during long-term diazotrophic cultivation. More research is needed to enlighten the mode of action of oligosaccharides in the regulation of PBP production in diazotrophic cyanobacteria.

Author Contributions

M.H. and T.D. performed the research, B.N. supervised, and T.Ç. designed the research and wrote the paper.

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